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## RESEARCH ARTICLE

# IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH ACCUMULATION OF SOLUBLE SILICON IN AEROBICALLY GROWN RICE

**Sajad Majeed Zargar[1*] and Muslima Nazir[2]**

[1]School of Biosciences and Biotechnology, Centre for Biodiversity Studies, BGSB University, Rajouri, Jammu and Kashmir – 185131 India
[2]Department of Botany, Jamia Hamdard University, New Delhi – 110062 India

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**ABSTRACT**

Silicon (Si) is a micronutrient, though not considered to be an essential nutrient for terrestrial plants but is often a major constituent of plant tissues. Apparently no other non-essential element is present in such consistently high amounts in the terrestrial plants. Si concentration in the plant tissues sometimes even exceeds the concentrations of nitrogen and potassium. It has been found to give resistance against various abiotic and biotic stresses mainly drought and blast disease respectively in case of rice. We estimated the soluble silicon content from fresh leaves at flowering stage of 51 diverse rice genotypes grown under aerobic conditions. Single marker analysis (SMA) and stepwise multiple regression analysis (SMRA) was done to find-out markers contributing for the silicon accumulation in rice. We identified a number of RAPD markers associated with the accumulation of soluble silicon in rice. Among the various RAPD markers, SMA established association of five RAPD markers among which maximum association was shown by $OPD3_{1000}$ (23.07%), followed by $OPB8_{700}$ (17.42%)while the SMRA showed $OPB8_{2000}$ and $OPC14_{1200}$ contributing more than 7% for accumulation of soluble silicon in leaves at flowering stage with positive parameter estimates. These markers can be used as an initial resource for identification and validation of tightly linked markers for this trait and later be used in molecular breeding program for improvement of rice crop. As silicon gives resistance against various abiotic (mainly drought) and biotic (blast disease) stresses in rice, hence can also be utilized in determining the resistance status of rice against these stresses.



*** Corresponding author:**
Sajad Majeed Zargar
E-mail: smzargar@rediffmail.com

## INTRODUCTION

Rice is one of the most important cereal crops. The production of rice is affected by various abiotic and biotic stresses among them drought is one of the major abiotic stress. Irrigated rice consumes about 3000-5000 liters of water to produce 1.0 kg of grain. However, looking at the trend with which the level of water table is depleting there is a need to develop the varieties which can perform well

under low moisture conditions. This idea has developed the concept called aerobic rice. The urgent need is to think in a holistic way to provide a solution for growing rice under lower moisture conditions. In the present study, 51 diverse rice genotypes were grown under aerobic conditions and accessed for accumulation of soluble Si.

Rice responses well to various nutrients and for the proper growth and higher yield of a plant there is an important need for the proper nutrient management. Silicon a micronutrient is beneficial element for the rice. Si has lot of beneficial effects (Epstein, 2001). It enhances the growth and yield, gives resistance against lodging, enhances photosynthesis, resistance against disease causing organisms, resistance to abiotic stress like salinity, drought and protection against temperature extremes. Si reduces the levels of several important diseases including blast, brown spot, sheath blight, leaf scald and grain discoloration (Seebold *et al*., 2000).

Si deposited in the tissues help to alleviate water stress by decreasing transpiration, improves light interception characteristics by keeping leaf blade erect, resistance against metal toxicity, bring remedies towards nutrient imbalances and other beneficial effects (Marschner, 1995; Epstein, 1994; Ma *et al*., 2002a and Hodson *et al.,* 2005). Present investigations have shown that Si induces the expression of various defensive genes. And it may be the soluble Si that can act like an inducer. Hence Si can rightly be called as a multifunctional micronutrient.

Molecular markers are of great value in applying genetic technologies to crop improvement such as marker assisted selection, gene pyramiding, QTL (Quantitative trait loci) mapping, targeted map based cloning of important genes, introgression of exotic germplasm, DNA fingerprinting of crops for detecting the markers associated with various traits. In the present study, 51 diverse rice genotypes were evaluated under aerobic conditions, their soluble silicon content in the leaves at flowering stage were estimated and finally RAPDs were employed to identify the markers associated with the accumulation of soluble silicon in aerobically grown rice.

# MATERIAL AND METHODS

### Plant material and field layout

51 diverse rice genotypes were used in the present study. The details of the genotypes are presented in Table 1. Rice genotypes were raised during summer (dry) season of 2005. The experiment was laid out in randomized complete block design (RCBD). The genotypes were grown under aerobic conditions without puddling and water stagnation, and the irrigation was given at an interval of 5 days. Direct line sowing was done with a spacing of 40cm between lines and 20cm spacing between plants. Every genotype was replicated twice.

### Soluble silicon estimation

Soluble silicon was estimated from the fresh leaves of flowering stage. The leaves were collected in ice from the field, and stored at $-80^0$C till further use. About (0.5g ) of fresh leaf samples were crushed in 10 ml of double distilled water using mortar and pastle, transferred to 40ml centrifuge tube and centrifuged at 8000 rpm for 10 min. Supernatant was taken in another graduated centrifuge tube and its volume was made 20 ml with 4% boric acid. The tubes were kept for few hours to allow debris to settle down. For estimation of soluble silicon content, 0.15ml of supernatant was taken to which, 6ml of 0.5N HCl (hydrochloric acid), 0.8ml of 10% $(NH_4)_6$ $Mo_7O_2$ (ammonium molybidate), 0.8ml of 20% tartaric acid, 0.8ml of reducing agent was added and diluted to 20.0 ml by adding 11.45ml of water, and allowed to develop blue colour (Ma *et al*., 2002b). For colour development samples were kept for at least 2 hr and absorbance was taken at 600 nm wavelengths. Estimation was replicated four times.

### DNA Extraction

The leaves from all 51 rice genotypes were collected separately and were dried in an oven for three days at $55^o$C to remove the moisture. These leaves were powdered using the mixer grinder, sieved and stored at room temperature for further use. The fine powder of leaves was used for the DNA extraction. DNA extraction was carried as per the method described by Porebski *et al.,* (1997)

with certain modifications. 100mg of leaf powder in 1.5ml of pre-warmed extraction buffer (100mM Tris pH 8.0, 20mM EDTA, 1.4M NaCl, 1% β-ME, 3% CTAB) was incubated in water bath at $65^0$C for 30 min with periodic shaking and centrifuged at 12,000 rpm for 20 min at $4^0$C. The supernatant was taken in a fresh tube and an equal volume of chloroform: iso-amylalcohol (24:1v/v) was added and vortexed gently, then centrifuged at 11,000 rpm for 10 min at $4^0$C. Aqueous phase was repeatedly washed with equal volume of *Chloroform: Isoamylalcohol* 24:1.

To the aqueous phase, equal volume of chilled iso-propanol and one tenth of the volume of 5M NaCl was added, mixed gently, kept at $-40^0$C for overnight to accentuate the precipitation of DNA. Centrifugation was done at 12,000 rpm for 20 min at $4^0$C to recover DNA pellet. The pellet was washed with 70% aqueous ethyl alcohol and air-dried. The pellet was dissolved in 100µl of TE buffer and incubated with 3µl (10mg/ml) of RNase overnight at $37^0$C. Washed with equal volume of phenol: chloroform: iso-amylalcohol (25:24:1v/v). DNA was precipitated by adding equal volume of chilled iso-propanol and kept at $-40^0$C for 2 hr, centrifuged at 12,000 rpm for 20 min to pellet the DNA. DNA pellet was dissolved in 300 µl of TE buffer and stored at $-40^0$C. DNA quantification was done at $OD_{260}$ nm and was diluted to a final concentration of 12.5ηg $\mu l^{-1}$ and 2µl of this DNA was used for the PCR amplification.

**Randomly Amplified Polymorphic DNA analysis**

Amplification was carried out in 20 µl reaction mixture containing 10X PCR buffer, 1mM dNTPs, 5pmol primers, 4 units Taq DNA polymerase and 25 ng of DNA template. Amplification reaction was carried out in a thermal cycler (MJ Research Inc. USA). The first cycle consisted of denaturation of template DNA at $94^0$C for 5 min, primer annealing at $36^0$C for 1 min and primer extension at $72^0$C for 2 min. In next 40 cycles, the period of denaturation was reduced to one minute while the time for primer annealing and extension remained as in the first cycle. The last cycle consisted of only primer extension at $72^0$C for 12 min. Total 120 random primers of arbitrary sequence (Operon Technologies Inc.) were screened by PCR analysis. Of the 120 primers screened, 14 primers produced strong, intense and unambiguous bands, and were selected for analyzing the samples. PCR products were separated on a 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml). 1X TBE buffer was used for preparation and running of the gel. The electrophoresis was carried out for 2.5 hr at 80V. The size of the fragments were determined by using (0.1-1Kb and 0.5-5Kb) DNA ladder/marker and the gel was visualized under ultraviolet light and documented using gel documentation system (Herolab GmbH Laborgerate).

**Data Analysis**

For the RAPD amplification profile, score 1 was given for the presence of a band and score 0 was given for the absence of band. SMA and SMRA was carried out using SAS (Statistical Analysis Software) v6. 12 program (SAS, 1989) and the regression values ($R^2$) were calculated to find the variability generated by these markers.

# RESULTS

Si has be identified as a micronutrient which helps in depleting various stresses. Rice being a Si accumulator, possesses this micronutrient in higher amounts however depending on the presence of the efficient Si transportes, there lies the variation in Si content not only between various genotypes but even with in a genotype. In the present study 51 diverse rice genotypes were grown in aerobic conditions. The soluble silicon from the fresh leaves of flowering stage was estimated as described in materials and methods.

**Concentration of soluble silicon among diverse rice genotypes**

A significant variation among the rice genotypes for the accumulation of soluble Si was found out . The concentration of soluble Si content among these 51 genotypes varied from 0.060% to 0.307% (Figure 1).

**Table 1. Diverse rice genotypes analyzed in the present study**

| Genotype No. | Designation | Source | Genotype No. | Designation | Source |
|---|---|---|---|---|---|
| 3 | B 6144F-MR-6 | A03WS-10 | 64 | IR 77298-5-6 | A03DS-03 |
| 5 | CT 13370-12-2-M | A03WS-09 | 67 | UPL RI 7 | A03DS-03 |
| 7 | CT 13382-8-3-M | A03WS-10 | 68 | Yunlu 29 | A03DS-03 |
| 8 | CT 6510-24-1-2 | A03WS-09 | 71 | IR 76558-156-4-1-3 | A04DS-02 |
| 13 | IR 47686-30-3-2 | A03WS-09 | 78 | DGI-196 | Binam |
| 15 | IR 55423-01 | A03WS-10 | 79 | DSL-89-3 | BG300 |
| 20 | IR 66421-062-1-1-2 | A03WS-09 | 80 | DSL-104-1 | Jhna349 |
| 22 | IR 68702-072-1-4-B | A03WS-09 | 83 | IR64-e7 | |
| 25 | IR 71524-44-1-1 | A03WS-09 | 85 | DSU-16-3 | Lemont |
| 26 | IR 71525-19-1-1 | A03WS-09 | 88 | RF-53-20 | Binam |
| 28 | IRAT 170 | A03WS-10 | 89 | DSU-4-4 | Feng-Ai-Zan |
| 32 | MARAVILHA | A03WS-09 | 90 | DSU-4-18 | Feng-Ai-Zan |
| 35 | PSBRC 82 | A03WS-09 | 91 | DSU-8-1 | Babaomi |
| 36 | UPL RI 5 | A03WS-09 | 92 | DSL-109-3 | MR 106 |
| 37 | UPL RI 7 | A03WS-09 | 94 | DSL-101-3 | Ptb33 |
| 41 | WAB 638-1 | A03WS-09 | 100 | DGI-143 | Type3 |
| 42 | WAB 96-1-1 | A03WS-09 | 102 | DGI 32 | STYH |
| 46 | IR 64 | A03WS-10 | 103 | DSU-4-7 | Feng-Ai-Zan |
| 47 | IR 70210-39-CPA-7-1-1-4-2 | A03WS-01 | 107 | DSL-78-10 | IR72 |
| 50 | IR 72875-94-3-3-2 | A03DS-03 | 108 | DSU-10-5 | Basmati |
| 52 | IR 74371-46-1-1 | A03DS-03 | 109 | DGI-155 | Type3 |
| 53 | IR 74371-54-1-1 | A03DS-03 | 110 | DSL-111-4 | MR 167 |
| 55 | IR 74371-78-1-1 | A03WS-01 | 115 | DGI 81 | BR24 |
| 56 | IR 74963-262-5-1-3-3 | A03WS-01 | 260 | IR 79907-B-145 | A04DS-03 |
| 57 | IR 75003-95-5-1-3 | A03WS-01 | 292 | IR 79907-B-177 | A04DS-03 |
| 59 | IR 77076-B-21-1-2 | A03WS-01 | | | |

**Table 2. Single Marker Analysis carried out for the soluble silicon content in rice leaves at flowering stage using RAPD markers**

| Marker | Partial $R^2$ | Total $R^2$ | PE | Prob.>F |
|---|---|---|---|---|
| $OPE13_{1900}$ | 0.0762 | 0.0762 | 0.0351 | 0.0500* |
| $OPE2_{1900}$ | 0.0781 | 0.0781 | 0.0422 | 0.0471* |
| $OPD3_{1000}$ | 0.2307 | 0.2307 | -0.0837 | 0.0004** |
| $OPC14_{1900}$ | 0.0896 | 0.0896 | 0.0493 | 0.0328* |
| $OPB8_{700}$ | 0.1742 | 0.1742 | -0.0689 | 0.0023** |

**RAPD markers associated with accumulation of soluble silicon in rice**

The DNA from all the 51 diverse genotypes was extracted as mentioned in material and methods section. This DNA was diluted to 25ηg/µl concentration of which 2 µl was used per reaction.

**Primer selection**

Among 120 random primers screened, fourteen primers OPA9, OPA11, OPB8, OPC11, OPD3, OPD5, OPD8, OPD9, OPC14, OPE1, OPE2, OPE4, OPE7 and OPE13 which produced intense, reproducible and more number of bands, were selected for the subsequent analysis. Figure 2 shows the RAPD profile of some selected genotypes using primer OPC 14.

**Association of RAPDs with soluble silicon content**

SMA revealed the association of five RAPD markers with the soluble Si content in the leaves at flowering stage (Table.2). Maximum association was shown by $OPD3_{1000}$ (23.07%), followed by $OPB8_{700}$ (17.42%), $OPC14_{1900}$ (8.96%), $OPE2_{1900}$

**Table 3. Stepwise Multiple Regression Analysis carried out for the soluble silicon content in rice leaves at flowering stage using RAPD markers**

| Marker | Partial $R^2$ | Total $R^2$ | PE | Prob.>F |
|---|---|---|---|---|
| $OPD3_{1000}$ | 0.2307 | 0.2307 | -0.0567 | 0.0004** |
| $OPB8_{700}$ | 0.1192 | 0.3499 | -0.1035 | 0.0047** |
| $OPC11_{750}$ | 0.0908 | 0.4407 | | 0.0082** |
| $OPD5_{800}$ | 0.0458 | 0.5310 | -0.1821 | 0.0417* |
| $OPB8_{2000}$ | 0.0403 | 0.5713 | 0.0629 | 0.0479* |
| $OPA9_{1400}$ | 0.0378 | 0.6965 | -0.1202 | 0.0313* |
| $OPC14_{1200}$ | 0.0334 | 0.7298 | 0.0655 | 0.0342* |
| $OPE2_{550}$ | 0.0283 | 0.7582 | 0.0805 | 0.0414* |
| $OPD3_{750}$ | 0.0354 | 0.7936 | -0.0291 | 0.0162* |
| $OPA11_{950}$ | 0.0244 | 0.8180 | | 0.0347* |
| $OPE13_{800}$ | 0.0234 | 0.8326 | -0.0477 | 0.0313* |
| $OPC14_{2150}$ | 0.0314 | 0.8640 | -0.1217 | 0.0074** |
| $OPE13_{1250}$ | 0.0223 | 0.8863 | 0.0729 | 0.0144* |
| $OPD9_{700}$ | 0.0283 | 0.9146 | 0.0276 | 0.0023** |
| $OPD5_{1000}$ | 0.0114 | 0.9260 | -0.0264 | 0.0338* |
| $OPE1_{1650}$ | 0.0108 | 0.9367 | -0.0392 | 0.0284* |
| $OPE2_{700}$ | 0.0109 | 0.9477 | | 0.0180* |
| $OPA9_{2400}$ | 0.0116 | 0.9573 | -0.0171 | 0.0078** |
| $OPD9_{2000}$ | 0.0068 | 0.9641 | 0.0263 | 0.0264* |
| $OPA9_{775}$ | 0.0068 | 0.9754 | -0.0306 | 0.0108* |
| $OPB8_{500}$ | 0.0062 | 0.9816 | 0.0264 | 0.0065** |
| $OPD8_{800}$ | 0.0044 | 0.9860 | | 0.0099** |
| $OPC14_{1600}$ | 0.0027 | 0.9887 | -0.0149 | 0.0246* |
| $OPE7_{500}$ | 0.0035 | 0.9922 | -0.0422 | 0.0041** |
| $OPC14_{600}$ | 0.0020 | 0.9942 | 0.0039 | 0.0112* |
| $OPD8_{600}$ | 0.0007 | 0.9968 | 0.0104 | 0.0460* |
| $OPD3_{350}$ | 0.0006 | 0.9979 | -0.0136 | 0.0417* |
| $OPD8_{1500}$ | 0.0003 | 0.9990 | -0.0042 | 0.0433* |

Note: **Significant at 0.01 probability level
*Significant at 0.05 probability level

(7.81%) and $OPE13_{1900}$ (7.62%). Among which four RAPD's $OPE13_{1900}$, $OPE2_{1900}$, $OPD3_{1000}$ and $OPC14_{1900}$ showed positive PE values. SMRA revealed the association of 28 markers, which together showed 85.23% association towards accumulation of soluble Si content in the leaves at flowering stage among which $OPD3_{1000}$ contributes 23.07%, $OPB8_{700}$ contributes 11.92% but both possess negative PE values.

The other markers which contribute for soluble Si accumulation in rice with positive PE values are $OPB8_{2000}$ (4.03%), $OPC14_{1200}$ (3.34%), $OPE2_{550}$ (2.83%), $OPE13_{1250}$ (2.23%), $OPD9_{700}$ (2.83%), $OPD9_{2000}$ (0.68), $OPB8_{500}$ (0.62), $OPC14_{600}$ (0.20) and $OPD8_{600}$ (0.07) (Table.3).

# DISCUSSION

Earlier repots mentioned that Si provides resistance against various stresses mainly due to its presence in cell wall which acts as a physical barrier, however its role in inducing various defensive genes had been elucidated (Fawe *et al*., 2001; Rodrigues *et al.,* 2003; Rodrigues *et al.,* 2004).. Hence the role of soluble Si comes in picture.

Rice is considered as Si accumulator and tends to actively accumulate Si to tissue concentrations of 5% or even higher (Epstein, 1994; Miyake and Takahashi, 1983). In our study the Si concentration varied from 0.060% to 0.307%, which is much lower than earlier reports. As in the

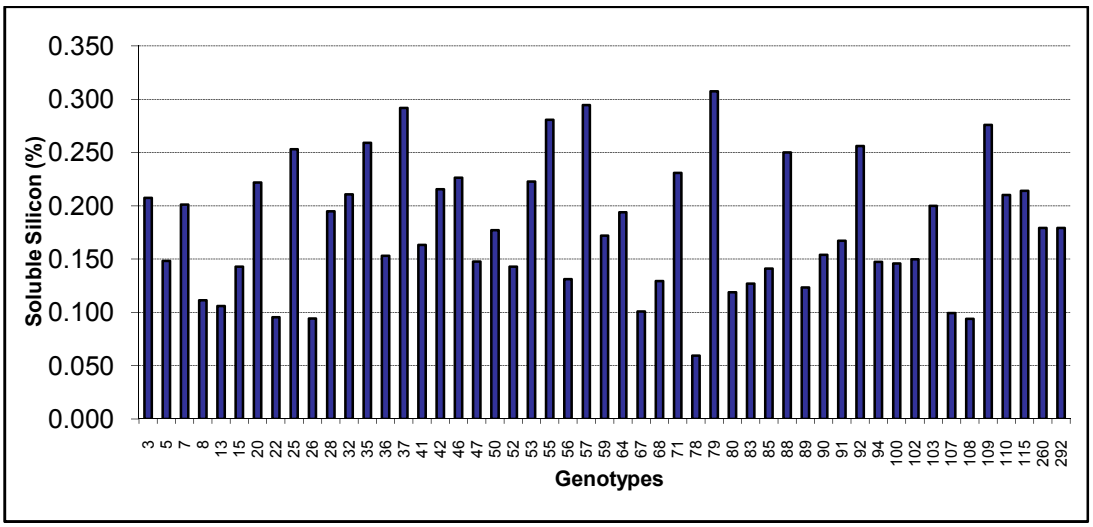


**Fig. 1. Percent soluble silicon in the leaves at flowering stage of rice genotypes**

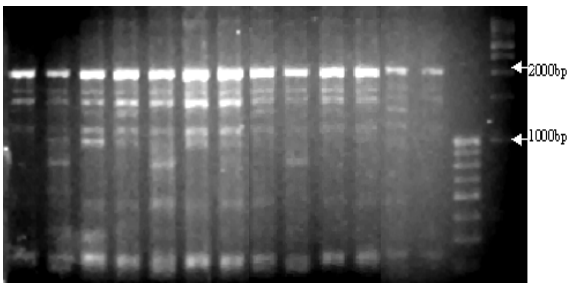


**Figure 2: RAPD gel profiles of selected Rice genotypes using Primer OPC14, M1 & M2 represents the Molecular ladders while different numbers represent various rice genotypes.**

present study we estimated only soluble Si content hence same was expected. The available Si appears to be very important for both robust growth and fungal disease resistance of rice (Winslow, 1992; Datnoff *et al.,* 1997). Si is known to provide resistance against various stresses and earlier studies have shown that there is a negative correlation between Si concentration and disease severity, however, this correlation is found much stronger within genotypes than among genotypes (Deren *et al*., 1994).

In the present study RAPDs were employed to identify the molecular markers associated with soluble Si content in rice. Using SMA and SMRA various markers were identified. These markers can be utilized as an initial resource to find out their actual linkage with trait of interest, validate them and use them in marker assisted selection for crop improvement. A similar work was done by Virk and co-workers in 1996, in which they used a set of 63 polymorphic RAPD primers to amplify 47 diverse Asian rice genotypes. They carried out multiple regression analysis to determine association between the presence and absence of individual marker and their results showed that 85% of the variation in the culm number and nearly all the variation in the flowering time could be explained by Regression models using sets of RAPD markers.

## Conclusion

A significant variation among the diverse rice genotypes for the soluble silicon content was found. The SMA and SMRA revealed association of large number of RAPD markers with the soluble silicon accumulation. These markers can be used as an initial resource to find out their actual linkage with trait of interest, validate them and use them in marker assisted selection for crop improvement. They can also be utilized for screening the rice germplasm for knowing their soluble Si content which will indirectly provide the information about their resistance potential against various biotic and abiotic stresses.

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